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Auxin Biosynthesis during Seed Germination in *Phaseolus vulgaris*[1]

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ABSTRACT

The relative roles of de novo biosynthesis of indoleacetic acid (IAA) and IAA conjugates stored in mature seeds (*Phaseolus vulgaris* L.) in supplying auxin to germinating bean seedlings were studied. Using ^2H oxide and 2,4,5,6,7-[^2H]L-tryptophan as tracers of IAA synthesis, we have shown that de novo biosynthesis of IAA, primarily from tryptophan, is an important source of auxin for young bean seedlings. New synthesis of IAA was detected as early as the second day of germination, at which time the seedlings began to accumulate fresh weight intensively and the total content of free IAA began to increase steadily. IAA conjugates that accumulate in large amounts in cotyledons of mature seeds may thus be considered to be only one of the possible sources of IAA required for the growth of bean seedlings.

It is believed that the major source of free IAA for young seedlings are IAA conjugates stored in the seeds during their maturation. This hypothesis was developed primarily for maize in which de novo biosynthesis of IAA in the seedlings of germinating kernels is not detectable and the major source of this hormone is ester conjugates (5, 19, 26). Bandurski and coworkers showed that the maize seedling is essentially a closed system, in which the level of IAA is regulated by transport from the endosperm, synthesis and hydrolysis of IAA conjugates, and IAA oxidation. However, even maize includes a few cultivars that may synthesize IAA de novo during seed germination (27); thus, the general applicability of the above hypothesis remains to be tested.

In legumes, the major portion of total IAA present in mature seeds is found in amide-linked conjugates (3, 9, 13, 18). In bean (*Phaseolus vulgaris* L.) seeds, amide conjugates account for more than 80% of the total IAA, and they consist predominantly of a series of immunologically related IAA peptides of 3 to 25 kD (6, 7, 16). We (11) showed that amide-linked IAA conjugates declined in bean cotyledons during the first 2 d of germination; however, as germination progressed, this decline slowed down and the level of IAA conjugates remained relatively high even after almost 1 week of germination. The content of free IAA, low in cotyledons, increased steadily in the axes during germination. Concomitantly with the increase in free IAA, the content of IAA conjugates also increased. Notably, IAA conjugates were also present in the seedlings from which the cotyledons were removed, and the ratio of free to conjugated IAA was even higher than in the axes of intact germinating seeds (11).

Based on these findings, it is possible to conclude tentatively that bean seedlings become independent from the cotyledons with respect to the supply of IAA during early stages of germination. To explore this possibility, we studied the role of de novo biosynthesis of IAA in supplying auxin to the axes of germinated seeds using ^2H_2O[3] and D_5-L-Trp as tracers of IAA biosynthesis. We used ^2H_2O in our studies because it is both a general label for which exact knowledge of the pathway of biosynthesis is not required and it is an invasive label not likely to be excluded specifically from any cellular compartments (25). ^2H_2O has been used before as a nonspecific tracer of de novo synthesis of the indole ring (17, 21, 27, 31). The incorporation of ^2H from ^2H_2O into any of five nonexchangeable positions, 2, 4, 5, 6, and 7, of the indole ring is an indication of de novo synthesis of IAA. As a second tracer of IAA biosynthesis, we used ^2H-labeled Trp because this amino acid is widely believed to be the precursor to IAA (15). The incorporation of five ^2H atoms into IAA isolated from plant material treated with D_5-L-Trp would be confirmation of de novo synthesis of IAA and would allow a distinction between the Trp and non-Trp pathways to IAA (31).

We show that both tracers of IAA biosynthesis are incorporated into IAA isolated from seedlings during bean germination and that the relative level of IAA synthesis increases with time of germination. Thus, we conclude that de novo synthesis of IAA primarily from Trp contributes significantly to the IAA pool of bean seedlings during seed germination.

MATERIALS AND METHODS

Plant Material

Bean seeds (*Phaseolus vulgaris* L., cv Bush Burpee, 1989 harvest, obtained from The Meyer Seed Co.[4], Baltimore) were

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[3] Abbreviations: ^2H_2O, deuterated water; GC-SIM-MS, GC-selected ion monitoring-MS; D_5-L-Trp, 2,4,5,6,7-[^2H]-L-Trp; MS medium, Murashige-Skoog medium.

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germinated aseptically at 26°C in the dark. Seeds were placed on several layers of disposable laboratory towels in 100- × 80-mm Petri dishes. The towels were wetted with 40 mL of either distilled water, 30% ^2H_2O (Cambridge Isotope Laboratories), or a water solution of D_5-L-Trp (MSD Isotopes; 98.5% isotope enrichment) at a concentration of 0.1 mg/10 mL of culture solution. Each experiment was repeated at least four times. Ten seeds were germinated in each culture dish.

After the specified time, the cotyledons and axes were separately removed from the germinating seedlings. Axes from this treatment will be referred to as "axes of intact seedlings." They were extensively washed in several changes of icy distilled water, and then their fresh weight was recorded. Axes, and in some experiments cotyledons, were immediately frozen in liquid nitrogen and then freeze-dried or directly ground in buffer. In Trp feeding experiments, an additional combination was included: the seeds were first allowed to imbibe in sterile water for 24 h, and then the axes were aseptically removed from the seeds and cultured on MS medium supplemented with 0.4 mg of D_5-L-Trp and solidified with 1.5% agar. With this treatment, the axes will be referred to as "isolated axes." The uptake of Trp in both experimental protocols was monitored by addition of 5-[^{3}H]-L-Trp (1.16 kBq/mL; 1 TBq/mmol, Amersham).

Monitoring the Incorporation of ^2H_2O and D_5-L-Trp into Plant IAA

The axes of seedlings incubated in 30% ^2H_2O were ground in a mortar to a fine powder using liquid nitrogen chilling and then homogenized in a mixture of 65% isopropanol/35% 0.2 M imidazole buffer, pH 7.0. Approximately 0.84 kBq of 5-[^{3}H]IAA (803 GBq/mol) was added during homogenization to facilitate peak detection during the purification procedure. After 1 h of equilibration at 4°C, insoluble materials were removed by centrifugation. Solvent was removed in vacuo, and the buffer residue was applied to a Baker SPE Amino (NH_2) disposable column conditioned with methanol, water, and 0.2 M imidazole buffer, pH 7.0. The column was first washed with 10 mL of distilled water and then eluted with 2 N HCl in methanol. Trp, which could interfere in the subsequent steps of the procedure, was not retained on the column; thus, it was removed with the efflux. The acidic methanol eluate containing free IAA and amide-bound conjugates was neutralized, methanol was removed in vacuo, and the residue was subjected to alkaline hydrolysis (7 N NaOH, 100°C, 3 h under water-saturated, O_2-free, nitrogen [8]) to replace exchangeable ^2H in the IAA molecule (27). The hydrolyzed sample was acidified with HCl and then desalted on a Fisher PrepSep C_{18} disposable minicolumn. The sample was then purified by HPLC on a Nova-Pak C_{18} reverse phase radial compression cartridge (5 × 100 mm, Waters). The mobile phase was 15% acetonitrile/water containing 1% acetic acid.

HPLC fractions containing IAA were evaporated in vacuo and methylated, and selected ion spectra were determined on a Hewlett-Packard 5890 (series II) GC/5971 A MSD. The GC was equipped with a DB-1701, 15-m × 0.237-mm i.d. fused silica capillary column (J & W Scientific); helium was used as a carrier gas at a flow rate of 1 mL/min. The injector temperature was 250°C, and the initial oven temperature was 140°C for 1 min followed by a ramp at 20°C/min up to 280°C. Monitored ions included the base peak of IAA methyl ester (the quinolinium ion, m/z 130) and its m + 1, m + 2, m + 3, and m + 4 isotopes (m/z 131, 132, 133, and 134, respectively), and the molecular ion (m/z 189) with its m + 1, m + 2, m + 3, and m + 4 isotopes (m/z 190, 191, 192, and 193, respectively). Seeds germinated on distilled water served as controls, and they were also used for analysis of the level of free and conjugated IAA by GC-SIM-MS with [$^{13}C_6$]IAA as an internal standard (14).

Samples from seedlings incubated with D_5-L-Trp were extracted, purified, and analyzed in the same way as for the ^2H_2O incubation experiments except that the isotopes at m + 5 and m + 6 of the base peak and molecular ion of methyl IAA (m/z 135–136 and 194–195) were included in the selected ions monitored.

Determination of the Labeling of Bean Axes Trp Pools with D_5-L-Trp

Axes grown in the presence of D_5-L-Trp were extracted with 65% isopropanol/35% 0.2 M imidazole buffer, pH 7.0, as described above. Approximately 0.84 kBq of [5-^{3}H]Trp was added to aid in peak detection. The homogenates were equilibrated for 1 h and centrifuged, isopropanol was removed in vacuo, and the buffer residue was purified as described earlier (23). The sample was applied to an 8-mL bed volume Dowex 50W-X8, 20- to 50-mesh, H^+ form, column. The column was washed with three bed volumes of distilled water and Trp eluted with 20 mL of 2 N NH_4OH. The eluate was evaporated to dryness, and traces of water were removed by multiple azeotropic distillations with anhydrous methanol and dichloromethane.

The dry residue was dissolved in a mixture of 2 mL of anhydrous methanol and 0.5 mL of acetic anhydride (Supelco) and incubated at 65°C for 1 h to form the *N*-acetyl methyl ester of Trp. After incubation, the reaction mixture was evaporated in vacuo to near dryness, dissolved in distilled water, and purified on a Baker SPE C_{18} disposable minicolumn conditioned with methanol followed by water. The *N*-acetyl methyl ester of Trp was eluted with acetonitrile. The sample was further purified by HPLC on a 3.9- × 150-mm Nova-Pak C_{18} column with 30% methanol/water as a mobile phase. Fractions containing *N*-acetyl methyl ester of Trp were combined and evaporated to dryness, and the dry residue was dissolved in ethyl acetate for GC-SIM-MS analysis. GC was done using essentially the same conditions as described above for IAA analysis except that the initial 140°C oven temperature (1 min) was followed by a ramp at 30°C/min up to 280°C. Monitored ions included the quinolinium ion (m/z 130) and the molecular ion of *N*-acetyl methyl ester of Trp (m/z 260) with their m + 1, m + 2, m + 3, m + 4, m + 5, and m + 6 isotope mass ions.

D_5-L-Trp was added at the beginning of isolation procedure as an internal standard for quantitative analysis of free Trp

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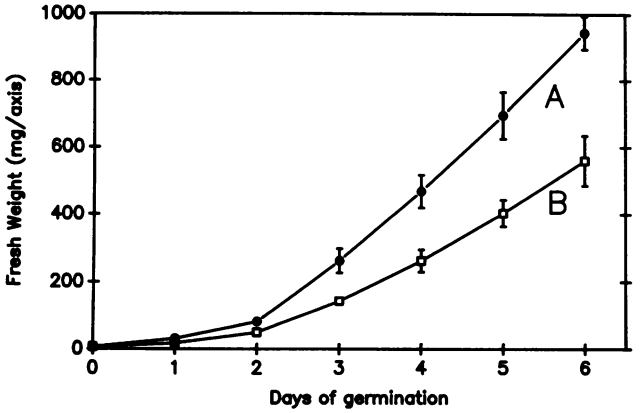

Figure 1. Accumulation of fresh weight in the axes of intact seedlings germinated on water (A) or 30% ^2H_2O (B). Vertical bars, when larger then the marked point, show the SD from the mean of three replicates, each containing 10 seeds.

in axes (either those removed from the seeds germinated on water or those isolated from imbibed seeds and cultured on MS medium). The unlabeled and labeled quinolinium and molecular ions (m/z 130, 135, 260, and 265) were analyzed by GC-SIM-MS. The amount of free Trp in the plant tissue was calculated using a modified isotope dilution equation, as described for the [$^{13}C_6$]IAA technique (14). A constant value R used for correction for the presence of natural abundances of heavy isotopes occurring in endogenous L-Trp and in fully substituted internal standard, D_5-L-Trp, was empirically determined to equal 1.16 (see equation in ref. 14).

RESULTS

Germination and Growth of Bean Seedlings in 30% ^2H_2O

We analyzed the incorporation of ^2H from ^2H_2O into IAA isolated from embryonic axes dissected from the bean seedlings germinated in 30% ^2H_2O for 3 or 6 d. As shown in Figure 1A, after 3 d of germination on water, axes had just begun to increase in fresh weight. Three days later, they had grown considerably. Growth of axes of intact seedlings incubated with 30% ^2H_2O was inhibited by 40 to 45%, as compared with water controls (Fig. 1B). This resulted in a 1- to 2-d retardation in their growth during the 3- to 6-d germination period, respectively.

Mass spectral analysis of methyl IAA isolated from the axes of intact seedlings germinated on 30% ^2H_2O (Fig. 2, Table I) showed significant incorporation of ^2H into nonexchangeable positions of the indole ring of IAA. This was indicated by more abundant heavy ions of the base peak (quinolinium ion) and molecular ion in mass spectra of IAA isolated from these seedlings. Mass distribution in the quin-

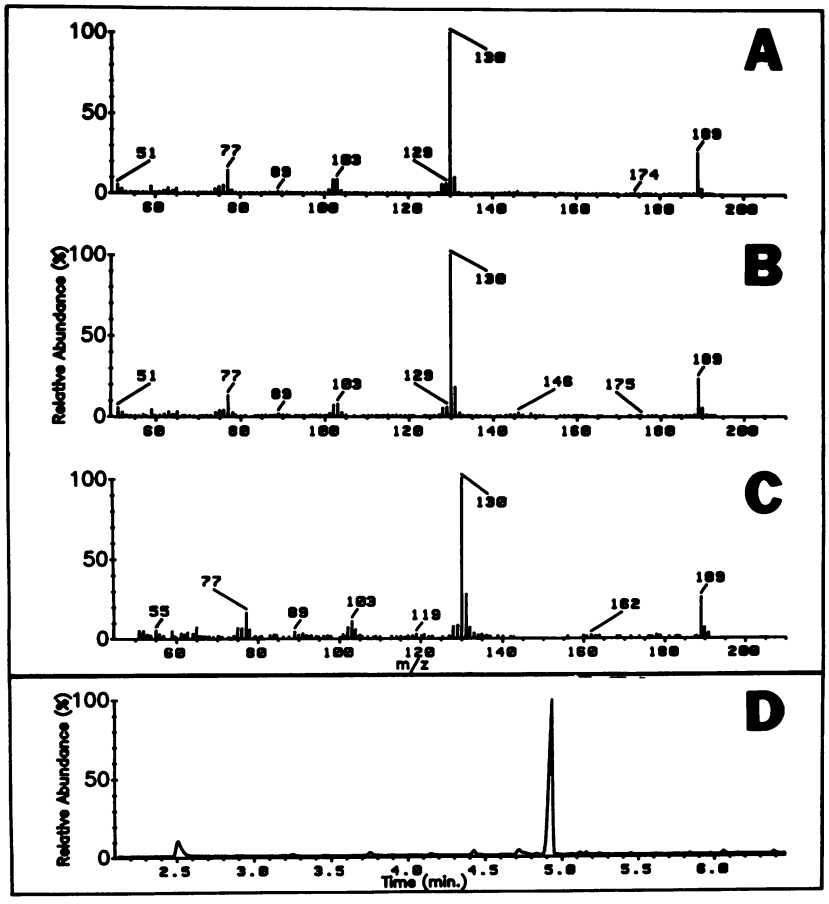

Figure 2. Average mass spectra of methylated IAA extracted from axes dissected from intact bean seedlings after 3 d of germination on water (A) or 30% ^2H_2O (B) or after 6 d on 30% ^2H_2O (C). Typical total ion chromatogram (the sum of monitored ions) for the IAA samples is shown (D). Each experiment was repeated at least four times, and the data are individual GC-MS analyses of representative samples.

olinium ion of native IAA (without added label) due to naturally occurring heavy isotopes should be: m/z 130 to 100%, 131 to 10.5%, and 132 to 0.4%. For molecular ion, they should be: m/z 189 to 100%, 190 to 12.9%, and 191 to 1.1% (28). Thus, 9.8% of the sum of ion abundances of the quinolinium ion region and 12.3% of the sum of molecular ion abundances are due to the normal abundance of heavy isotopes (primarily ^{13}C) in nature. After 3 d of germination on 30% ^2H_2O, the percentage of heavy isotopes detected in the cluster of the quinolinium ion (m/z 130 and m + 1, m + 2, and m + 3) was 17.0%, and after 6 d of germination, this value increased to 28.1%. After correcting for the occurrence of natural isotopes in the unlabeled quinolinium ion (9.8%), we found that after 3 d of germination 7.2% (17.0–9.8%) of IAA molecules contained one or more ^2H atoms in the indole ring. Incorporation of ^2H into plant IAA increased with time of germination, and after 6 d the percentage of molecules with ^2H increased to 18.3% (28.1–9.8%). Mass spectral analysis of methyl IAA isolated from water-grown seedlings showed good agreement of relative abundances of ions at masses 131 and 190 with those of IAA standards.

We used 30% ^2H_2O in our studies to label precursors of IAA biosynthesis because a 30% enrichment of water in ^2H was found to be effective for use in the studies of biosynthetic pathways with nearly normal plant growth (27). Thus, to calculate the actual percentage of newly synthesized IAA, we compared incorporation of ^2H found in our experiments to that which is theoretically possible at this isotope enrichment and with five stable positions of the indole ring available. This can be calculated using the equation that describes the distribution of isotopic abundance at a given enrichment of the precursor for each partially or totally enriched product:

$$M_a = (E_1 + E_2)^n$$

where M_a is the total abundance of the ion of the synthesized product (equals 1), E_2 shows the isotope enrichment of the precursor, E_1 relates to the concentration of unsubstituted precursor, and n gives the number of atoms that could possibly be incorporated into the synthesized molecule (24). We assume for this calculation that essentially 100% of the IAA molecules are synthesized de novo.

The enrichment of ^2H in water used in our experiments was 30%; thus, $E_2 = 0.3$ and $E_1 = 0.7$. The number of atoms (n) that could possibly be incorporated into the indole ring is 5. Thus,

$$M_a = (0.7 + 0.3)^5.$$

Expanding this binomial equation gives the fraction of molecules partially to fully labeled with ^2H, plus the abundance of unsubstituted molecules. The equation does not take into account the contribution of naturally occurring heavy isotopes. Table II shows values calculated for the quinolinium ion distribution of theoretical incorporation of heavy isotopes expressed as a percentage of the total ion abundance. Thus, using 30% ^2H_2O as a tracer of IAA biosynthesis, if all of the IAA is produced de novo from newly synthesized precursors and the system is at steady state, one can expect only 16.8% of total abundance to be related to unsubstituted molecules (m/z 130). About one-third of the IAA molecules would incorporate either one (m/z 131) or two (m/z 132) ^2H atoms. Total substitution (all five positions in the indole ring occupied by ^2H) could be expected in only 0.2% of the molecules. Together, 83.2% of total abundance of the quinolinium ion would be related to the ^2H incorporated in one or more positions of the indole ring. We found an increase of 7.2 and 18.3% in the abundance of heavy ions in the cluster of the quinolinium ion after 3 and 6 d of germination, respectively. This gives 8.7 and 22.0% of the theoretically possible incorporation of ^2H into the indole ring at 30% ^2H enrichment. These numbers, thus, reflect the actual percentage of de novo synthesized IAA during the period studied.

Incubation of the Axes of Intact Seedlings or Isolated Axes in the Presence of D_5-L-Trp

Mass spectral analysis of IAA isolated from axes of intact seedlings germinated in the presence of D_5-L-Trp showed the

Table I. *Mass Spectral Analysis of Methylated IAA Isolated from Axes of Seedlings Germinated for 3 or 6 d on Either Water or 30% ^2H_2O*

Mass/Charge (m/z)	Relative Abundance[a]			
	IAA Standard	H_2O 3 d	30% ^2H_2O	
			3 d	6 d
130	100[b]	100	100	100
131	10.8	10.5	18,0	27.5
132	0.7	0.5	2.0	7.6
133	0.0	0.0	0.2	2.6
189	100	100	100	100
190	12.7	12.4	20.3	26.6
191	1.1	1.0	2.4	10.4
192	0.1	0.1	0.0	0.0

[a] The quinolinium (m/z 130) and molecular (m/z 189) ions of methyl ester IAA each normalized to 100%; m + 1, m + 2, and m + 3 ions are expressed as the percentage abundance relative to m (m/z 130 or 189). [b] Results represent the means from at least four replicate experiments. Variation among samples was <5%.

incorporation of labeled Trp into IAA. This was shown by the presence of the m + 5 isotope ion of the quinolinium (m/z 130) and molecular (m/z 189) ions in the spectrum of methylated IAA (Table III). The spectrum of unlabeled methyl ester of IAA has no peaks at m/z 135 and 194; thus, any abundance detected is a direct measure of conversion from the applied labeled Trp. The ratio of m/z 130 to 135 and the ratio of m/z 189 to 194 is the ratio between the IAA made or retained from unlabeled precursors and that made by conversion of D_5-L-Trp.

The percentage of IAA molecules labeled with D_5-L-Trp, expressed as a ratio of abundance of m/z 135 ion to a sum of abundances of m/z 130 and 135, was low in the axes of intact seedlings: 3.8 and 5.0% of the molecules labeled after 3 and 6 d of germination, respectively. To determine whether such a low incorporation of Trp into IAA was an indication of a very slow rate of IAA biosynthesis or was only related to a dilution of the label by endogenous Trp, the axes were isolated from the seedlings and were cultured on agar supplemented with D_5-L-Trp as described in "Materials and Methods" (isolated axes). This technique removed the cotyledons, which are the major source of Trp in a young seedling. After 6 d of germination (or, in the case of isolated axes, 1 d of imbibition and 5 d of growth on agar), we compared labeling of the endogenous Trp pool with D_5-L-Trp (Fig. 3) and incorporation of the label into IAA (Fig. 4) in both the axes of intact seedlings and isolated axes grown on agar. At the same time, the levels of Trp as well as IAA were determined. Additionally, uptake of labeled Trp was compared in both situations using [5-H^3]Trp as a tracer.

Data from these experiments are summarized in Table IV. Growth of isolated axes was greatly reduced in comparison with axes of intact seedlings to the extent that isolated axes contained about 34 times less free Trp (3.6 versus 121.7 μg/axis) and about 30 times less free IAA (0.5 versus 15.0 ng/axis). The total amount of IAA per axis was about 10 times lower (4.0 versus 42.3 ng/axis), thus yielding a similar concentration because the ratio of fresh weight accumulated in the axes under these two experimental conditions differed also by almost an order of magnitude (104 versus 945.0 mg/axis). Axes of intact seedlings took up 2.2 μg of labeled Trp per axis after 6 d of culture, and this constituted 1.8% of the total free Trp pool of the seedling. Isolated axes took up almost twice as much labeled Trp (4.0 μg/axis), and this accounted for more than 50% of the total free Trp pool.

Mass spectral analysis of endogenous Trp of axes grown in the presence of D_5-L-Trp in these two treatments also showed a dramatic difference in the labeling (Fig. 3). Calculation of the ratio of m/z 135 ion relative to the sum of m/z 130 and 135 ions revealed that in the axes of intact seedlings only 1.7% of extracted Trp was labeled with five ^2H atoms, whereas in the isolated axes grown on agar almost 60% of extracted Trp was labeled. Thus, a low incorporation of Trp

Table III. *Mass Spectral Analysis of Methylated IAA Isolated from Bean Axes Grown in the Presence of D_5-L-Trp*[a]

Mass/Charge (m/z)	Relative Abundance[b]		
	Axes of intact seedlings		Isolated axes grown on agar
	3 d	6 d	
130	100[c]	100	100
135	3.8	5.0	60.8
189	100	100	100
194	4.9	6.6	65.8

[a] Axes of intact seedlings were removed for analysis after 3 or 6 d of germination, whereas axes grown on agar were isolated from seeds allowed to imbibe for 24 h and then cultured on MS medium containing 1.5% agar and supplemented with D_5-L-Trp for another 5 d. [b] The quinolinium (m/z 130) and molecular (m/z 189) ions of methyl IAA were each normalized to 100%; m + 5 ion is expressed as a percentage abundance relative to m (m/z 130 or 189). [c] Results represent the means from four replicate experiments. Variation among samples was <5%.

Table II. *Incorporation of ^2H from ^2H_2O into Stable Positions of the Indole Ring of IAA from the Axes of Intact Bean Seedlings as a Measure of de novo Biosynthesis*

	Mass Distribution of Quinolinium Ion Region						Total heavy ions	Total heavy ions corrected for natural isotopes presence	De novo synthesis of IAA
	Unlabeled	Related to heavy isotopes presence							
	m (m/z 130)	m + 1 (m/z 131)	m + 2 (m/z 132)	m + 3 (m/z 133)	m + 4 (m/z 134)	m + 5 (m/z 135)			
					%				
Unlabeled IAA	90.2	9.5	0.3				9.8		
Theoretically calculated for 100% de novo synthesis incorporation of ^2H from 30% ^2H_2O	16.8	36.0	31.0	13.2	2.8	0.2	83.2		
IAA from axes of seedlings[a] grown on 30% ^2H_2O for:									
3 d	83.0	15.0	1.7	0.2	0.1		17.0	7.2	8.7
6 d	71.9	19.8	5.4	1.9	1.0		28.1	18.3	22.0

[a] Calculations of ^2H incorporation into plant IAA were based on data presented in Table I.

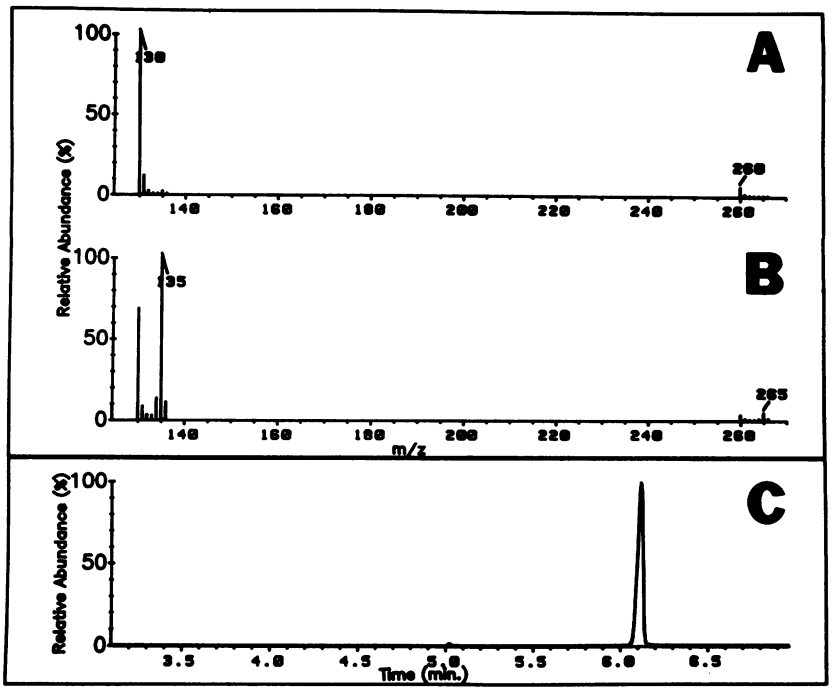

Figure 3. Average selected ion mass spectra of *N*-acetyl methyl ester of Trp extracted from bean axes germinated with 10 μg of D_5-L-Trp/mL in the culture medium. A, Axes of intact seedlings germinated for 6 d on D_5-L-Trp solution. B, Axes isolated from seeds allowed to imbibe for 24 h and then cultured for another 5 d on MS medium (1.5% agar) supplemented with D_5-L-Trp (isolated axes). C, Typical total ion chromatogram for the Trp samples. Each experiment was repeated at least four times, and the data are individual GC-MS analyses of representative samples.

to IAA in the axes of intact seeds is related to a low labeling of the Trp pool with D_5-L-Trp. Much higher labeling of the Trp pool can be achieved with isolated axes grown on agar (59.3%), and this is reflected in high incorporation of Trp into IAA (60.8%).

DISCUSSION

Our experiments show clearly that bean seedlings synthesize IAA de novo in the first days of germination. Using either general or specific precursors of IAA biosynthesis labeled with ^2H, we have detected new IAA synthesis as early as the third day of germination. Because seedling growth was inhibited in 30% ^2H_2O by almost 50% relative to water control (Fig. 1), which meant almost 1 d of retardation in the seedling growth during the 3-d germination period, one can assume that this synthesis actually occurred at an earlier stage of germination relative to the water controls. We (11) showed previously that the levels of both free and amide-conjugated IAA begin to increase after the first day of germination, with the most dramatic increase observed between the second and third days of germination (11). This increase in the level of IAA parallels the increase in the accumulation of fresh weight in the axes of germinating seeds (Fig. 1). The incorporation of the label from ^2H_2O into IAA follows the same pattern as the increase in both fresh weight and free and conjugated IAA. Thus, a steady increase in the IAA content in bean seedlings during germination seems to be related to increasing de novo IAA synthesis. The comparison of incorporation of ^2H_2O into IAA in the axes and cotyledons revealed that new IAA synthesis was limited to the axes, and it was practically undetectable in the cotyledons (data not shown). The rate of ^2H incorporation may also depend on the age of the seeds. We noticed that ^2H_2O incorporation in the seeds from the previous harvest year was almost as great after 3 d as in the seeds from the most recent harvest after 6 d of germination (data not shown).

Our observation of new IAA biosynthesis in the early stages of the growth of bean axes contrasts with data reported for maize (sweet corn) in which ester IAA conjugates accumulated in great quantities in the endosperm were practically the only source of free IAA for the shoots of germinating seedlings (5, 19, 26, 27). However, Iino and Carr (20) reported that in an Australian field corn, GH 390, IAA present in the mesocotyl may originate from a source other than IAA ester conjugates, and they attribute this to biosynthesis. Weiler and Wishnewski (29) also attributed the auxin source in coleoptile segments of maize to biosynthesis. Unfortunately, their data are difficult to interpret because they did not examine amide IAA conjugates, and our work has shown that field corn, GH 390, contains high levels of amide bond auxin (R. Baraldi and J.D. Cohen, unpublished).

The competence of maize seedlings for IAA biosynthesis seems clear (31); however, the expression of this competence may depend on the availability of IAA conjugates, specifically ester conjugates, as a source of the required IAA. The bean seeds used in our studies contain large amounts of IAA conjugates, especially amide-linked conjugates: about 600 ng · g^{-1} fresh weight or 150 ng/seed at full seed maturity (8, 9). Their levels decline in the first days of germination but remain relatively high through the later period of germination (11). Although auxin conjugates might be an important source of IAA in the very early stages of germination in bean and they could, at later stages, supplement the IAA required for growth, it is obvious that the shoots of bean seedlings not only are competent for IAA biosynthesis but even in the first days of germination they produce IAA biosynthetically. The difference in the source of free IAA in the young seedlings

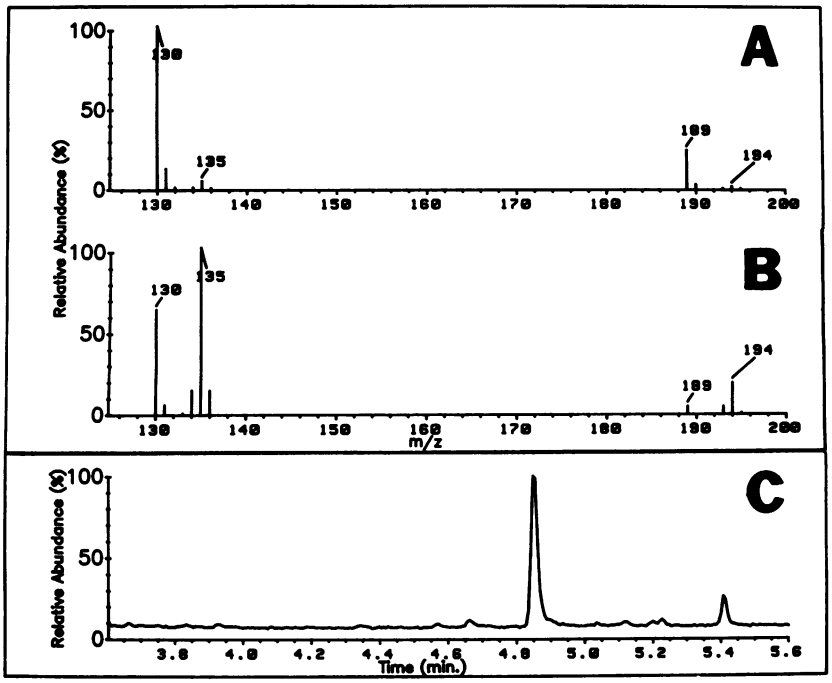

Figure 4. Average selected ion mass spectra (A and B) and typical total ion chromatogram (C) of methylated IAA extracted from the axes grown with 10 μg of D_5-L-Trp/mL in the culture medium. A and B are as described in Figure 3. Each experiment was repeated at least four times, and the data are individual GC-MS analyses of representative samples.

of sweet corn and bean may be explained not only by a different type of IAA conjugate present in maize kernels (mostly esters) and bean seeds (mostly amide-linked conjugates) but also by their location in structurally different types of storage tissue. The availability of IAA conjugates as a source of free IAA in a young seedling may depend not only on their chemical characteristics (for example, their stability) but also on how readily they can be transported from the storage tissue to the axes.

Our ^2H_2O-labeling results in bean are similar to those reported for 5-d-old maize shoots in which about 22% of the IAA molecules were labeled with ^2H under similar experimental conditions (27). In our experiments, we noted 18.3% ^2H incorporation, but we analyzed whole axes with roots, which were shown to incorporate much less ^2H into IAA than shoots (26). Tomato shoots, excised from 1-month-old plants and incubated using the same concentration of ^2H_2O, incorporated similar levels of ^2H into their IAA, 17.3% after 10 h of incubation (17). The percentage of IAA molecules incorporating different numbers of ^2H atoms as related to the total labeled molecules was practically the same for all three plants, i.e. approximately 60% of the molecules had one ^2H atom, 30% contained two ^2H atoms, and 9% had three atoms incorporated. This suggests, as might be expected, that IAA synthesis occurs through similar early steps in these different plants.

The percentage of IAA molecules that incorporated ^2H from heavy water refers only to IAA synthesized from precursors that have been themselves synthesized de novo using ^2H-labeled water, and it does not take into account IAA synthesized de novo from preexisting precursors of IAA biosynthesis. Trp, which is believed to be one of the possible IAA precursors, was abundant in the axes of germinating seedlings (Table IV). Its level of approximately 130 μg/g of

Table IV. *Comparison of the Relationship between Trp and IAA in the Axes of Intact Bean Seedlings and Isolated Axes Cultured on Agar*[a]

Combination	Fresh wt[b]	Trp Level[b]	Uptake of Deuterated Trp from Medium[b]	Free IAA Level[b]	Total IAA Level[b]	Labeling of Endogenous Trp Pool with D_5-L-Trp[c]	Incorporation of D_5-L-Trp to IAA[c]
	mg/axis	*μg/axis*	*μg/axis*	*ng/axis*	*ng/axis*	%	%
Axes of intact seedlings (A)	945.0	121.7	2.2	15.0	42.3	1.7	5.0
Isolated axes (B)	104.0	3.6	4.0	0.5	4.0	59.3	60.8
Ratio A/B	9.1	33.8	1/1.8	30.0	10.6	1/34.9	1/12.2

[a] All data were obtained after 6 d of germination. In both cases, the axes were grown in the presence of D_5-L-Trp. [b] Values representing the levels of Trp and IAA, fresh weights, and Trp uptake are means from three to six experiments and variation among samples did not exceed 10%. [c] Based on mass spectral analysis of either Trp or IAA and expressed as a percentage of m/z 135 ion abundance relative to the sum of m/z 130 and 135 abundances. Results represent the means from four replicate experiments. Variation between samples was <5%.

fresh weight was much higher than the level reported for older bean seedlings (14 μg/g of fresh weight [12]) or, for example, in mature and senescing *Ricinus communis* leaves (30–80 μg/g of fresh weight [1]). The level of Trp in isolated axes grown on agar (approximately 35 μg/g of fresh weight) was within the range of values reported in the literature for Trp in plant tissues.

The probable source of Trp noted at high levels in the axes of intact germinating seedlings is the proteolysis of storage proteins. Trp will not move actively in excised tissue segments commonly used in auxin transport studies; however, it is translocated in intact seedlings when applied to the storage part of seeds, for example, cotyledons of *Phaseolus coccineus* (30). IAA synthesized from Trp released from storage proteins would not be labeled in the ^2H_2O experiments. We have shown in experiments with D_5-L-Trp that IAA is synthesized primarily from Trp in isolated bean axes grown on agar. We found that the percentage of IAA labeled with deuterated Trp (60.8%) corresponds well with the 60% labeling of the endogenous Trp pool. In addition, we observed the same labeling of the plant Trp pool with radioactive Trp taken up by bean axes from the culture medium. Also, the level of free IAA in isolated axes was reduced to almost the same degree as the level of Trp. These data show that the bulk of IAA in bean originates from Trp rather than from other precursors or stored conjugates.

There is a possibility that the Trp pathway of IAA synthesis was activated by removal of the cotyledons, which contain the bulk of the IAA conjugates. However, predictably low enrichment of the plant Trp pool with D_5-L-Trp in the axes of germinating intact seedlings explains the observed low ^2H incorporation into IAA. Incorporation into IAA was actually slightly higher than the enrichment of the bulk Trp pool. This higher labeling of IAA with D_5-L-Trp than the plant Trp pool in the axes of intact seedlings may be a result of the preferential use of unlabeled Trp in cytoplasmic protein synthesis.

Trp has long been considered to be the immediate precursor to IAA (15), but it also was suggested (2, 22) and has recently been shown in studies with a Trp auxotroph mutant (31) that IAA biosynthesis can proceed without Trp as a metabolic intermediate. On the basis of these findings, it was proposed that the Trp pathway was only a minor contributor to IAA biosynthesis (31). Our experiments show that this is not always the case. We have shown that Trp is an important precursor of IAA in the axes of germinating bean seedlings. Our findings are in agreement with the report by Black and Hamilton (12), who observed that excised bean shoots convert radioactive indole to IAA with Trp as the apparent intermediate.

The marked increase of IAA conjugates in the total IAA pool in isolated axes in comparison to the axes of intact seedlings could be discussed in terms of the role of these conjugates in the control of IAA concentration in the plant. Bandurski et al. (5) proposed that conjugation of hormones and their subsequent hydrolysis may be important in maintaining a steady-state concentration of hormone in plant tissues responsive to environmental conditions. Bandurski and coworkers (4) showed that the decrease in the level of free IAA following exposure of maize seedlings to conditions resulting in reduction of their growth rate was accompanied by an increase in the level of ester IAA conjugates.

Our results support the hypothesis that IAA conjugates are a component of a system for homeostatic control of this hormone in plant tissues. In our experiments, we observed the same total IAA concentration per fresh weight unit in both axes of intact seedlings and isolated axes grown on agar; however, the ratio of free to conjugated IAA was different. In the isolated axes grown on agar, whose growth was greatly reduced after cotyledon removal, we recorded a shift toward amide conjugates in the total IAA pool. We (10) showed before that IAA conjugates were hydrolyzed to yield free IAA when applied to excised bean stem sections. Thus, conjugation of IAA in bean seedlings appears to be another example of the involvement of conjugates in hormonal homeostasis in relation to growth control.

In summary, we have shown that de novo biosynthesis of IAA, primarily from Trp, is an important source of auxin for young bean seedlings. IAA conjugates accumulated in great quantities in cotyledons of mature seeds may thus be considered to be only one of the possible sources of IAA required for the growth of bean seedlings. The pattern of changes in level of IAA conjugates during germination, especially after cotyledon removal, suggests that their metabolic role is primarily to function in the homeostatic regulation of IAA levels in bean seedlings.

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